The interactive effect of elevated CO_2 and herbivores on a nitrogen-fixing plant

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Abstract: Many studies have found that future predicted CO_2 levels (< 800 ppm) can increase plant mass but dilute N content in leaves, impacting antiherbivore compounds. Nitrogen-fixing plants may balance leaf C:N ratio under elevated CO_2, counteracting this dilution effect. The aim of this study was to look at how nitrogen-fixing plants grow and respond to herbivore damage at different CO_2 levels. *Alnus incana* ssp. *rugosa* was grown at 400, 800, or 1600 ppm CO_2 in soil collected from the field, inoculated with *Frankia* and exposed to herbivores (*Orgyia leucostigma*). Elevated CO_2 increased nodulated plant biomass and stimulated the nitrogen fixation rate in the early growth stage. However, nitrogen-fixing plants were not able to balance the C:N ratio under elevated CO_2 after grown 19 weeks. When plant were grown at 400 and 1600 ppm CO_2, herbivores preferred to feed on leaves of nodulated plants. At 800 ppm CO_2, nodulated plants accumulated more total phenolic compounds in response to herbivore damage than plants in the non-*Frankia* and non-herbivore treatments. Our results suggest that plant leaf defence, not leaf nutritional content, is the dominant driver of herbivory and nitrogen fixing plants have limited ability to balance C:N ratios at elevated CO_2 in natural soil.

Keywords: elevated CO_2, nitrogen-fixing plants, herbivores, total phenolic compounds, C:N ratio.

1. Introduction

The growing conditions when major plant evolutionary events took place are quite different from the present. Vascular plants originated and adaptively radiated during the Early Silurian period ca. 440 million years ago [1]. The conditions during this period included an atmospheric CO_2 level of 3300-3600 ppm [2]. The evolution of nitrogen-fixing plants (i.e. the symbiosis with nitrogen fixation bacteria involving the formation of root nodules) occurred during the rapid expansion of flowering plants in the late Cretaceous *ca.*100 MYA [3]. During this time, atmospheric CO_2 was *ca.* 1600 ppm, around four times the present atmospheric level [3–6]. The nitrogen-fixing symbiosis was likely more advantageous under conditions of ancient CO_2 levels. Many studies have found that future predicted CO_2 levels (< 800 ppm) increases plant N fixation rates [7–9]. This increase has been shown to allow plants to maintain their leaf C:N ratio, whereas non nitrogen fixing plants show increases in leaf C:N ratio with increasing CO_2 levels [10]. However, these studies often provide plants with additional nutrients, especially P, which can stimulate an increase in nodule number or mass, but may not represent how nitrogen fixing plants respond under natural soil conditions. Therefore, it is possible that the decrease in atmospheric CO_2 over geological time restricted the success of existing, and evolution of new nitrogen-fixing species [6,11]. This would also explain the loss of the nitrogen-fixing trait that has occurred within the clade of nitrogen-fixing plants [11–13].

Many studies simulating predicted CO_2 level increases in the coming decades have shown that small increases in atmospheric CO_2 increase plant growth. Elevated atmospheric CO_2 levels increase plant water use, allowing for increased carbon assimilation [14]. However, plant performance does not increase indefinitely with an increasing atmospheric CO_2 concentration [15,16]. For example, it has been shown that elevating CO_2 from 340 ppm enhanced non-nitrogen fixing plant seed yield by 30% to 40%, peaking at 1200 ppm CO_2, and then decreasing as CO_2 levels increase to 2400 ppm [15]. Increased carbon assimilation and storage of carbohydrates also have the effect of diluting other nutrients, such as nitrogen, within plant tissues [10,17,18]. This increase in leaf C:N ratio of tissues can alter plant interactions with other organisms and ecosystem processes.

There has been extensive work on plant interactions with herbivores at predicted future increases in CO_2 levels on plants in general, with several studies including nitrogen-fixing plants

[19,20]. While a higher leaf C:N ratio makes plant leaves less nutritious for herbivores, they may compensate by increasing their consumption rate to meet growth requirements [17,21]. Elevated atmospheric CO_2 can also increase plant carbon-based defensive compounds (e.g., phenolic compounds) and decrease nitrogen-based defensive compounds due to the dilution of N in leaves [17,22].

Since the ability of plants to form a nitrogen symbiosis can alter their response to CO_2 levels, it may also alter their interaction with herbivores. Nitrogen-fixing plants can reduce plant tissue consumption by herbivores at elevated CO_2 levels [20]. Nitrogen-fixers can utilize C-based or N-based defensive compounds against herbivores [17,23] and the balancing of C:N ratio can affect the production of these defensive compounds. A study on nitrogen-fixing black alder showed leaf carbon-based (total phenols) and nitrogen-based (peroxidases and polyphenol oxidases), antiherbivore compounds increased in response to previous herbivore damage [23]. On the other hand, herbivores can strongly limit some nitrogen fixers' abundance and prevent their dominance in N-poor soils due to the herbivores' preference for nitrogen-fixing plants with a high leaf nitrogen content [24]. Elevated CO_2 (700 ppm) has been shown to shift herbivore preference to non-N fixers (cotton), being consumed three times more than N fixers (alfalfa) [19]. This suggests that elevated CO_2 can trigger a stronger effect on the herbivore-induced response of N fixers compared with non-N fixers when plants are pre-damaged.

We hypothesize that when nitrogen-fixing plants first evolved in the late Cretaceous under high CO_2 levels, they had a different relationship with herbivores compared with the present. No studies have examined the interaction between nitrogen-fixing plants and herbivores under ancient levels of CO_2. The interaction between nitrogen-fixing plants and herbivores at a future level of CO_2 may not reflect interactions in the Cretaceous since we know that plant performance peaks below the ancient level of CO_2. The aim of this study is to look at the effects of future predicted (800 ppm) and Cretaceous period (1600 ppm) CO_2 levels on nitrogen-fixing plant growth, leaf C:N ratio, carbon-based and nitrogen-based antiherbivore compounds. We compared nitrogen-fixing plants that formed nitrogen-fixing nodules to plants prevented from doing so. We also examined how growing plants at these CO_2 levels affect preference for feeding on leaves of nitrogen-fixing or non-nitrogen fixing plants and the response of plants to feeding damage. We predicted that: 1. nitrogen-fixing plants can balance leaf C:N ratio under elevated CO_2 compared to non-fixing plants, 2. as CO_2 levels increase, carbon-based antiherbivore compounds will increase, and nitrogen-fixing plants will be able to maintain the levels of nitrogen-based antiherbivore compounds, 3. plants that accumulate more carbon-based or nitrogen-based antiherbivore compounds will have less herbivore damage, 4. herbivores prefer to feed on leaves from nitrogen-fixing plants when given a choice but, 5. herbivores consume more leaf tissue as the C:N ratio increases.

2. Results

2.1. Plant biomass

Plants that developed nodules were 55 times larger than plants without (Figure 1). Nitrogen-fixing plants also increased biomass in response to increasing CO_2, being ca. 1.5 larger at the 800 ppm and 1600 ppm CO_2 level than plants grown at 400 ppm CO_2. Non-nodulated plants showed no response to increasing CO_2 ($F = 51.84$, $P < 0.0001$, for the inoculation treatment by CO_2 treatment interaction). A non-linear (quadratic) model of nodulated plant biomass versus CO_2 level predicted that plant biomass would peak at 1137 ppm ($F = 39.50$, $P = 0.003$, $R^2 = 0.63$) (Figure S1). Herbivory alone ($F = 0.002$, $P = 0.96$), herbivory crossed with CO_2 level ($F=1.41$, $P=0.24$), herbivory crossed with Frankia inoculation ($P=0.94$, $F=0.005$), or the interaction among all three factors ($F=1.49$, $P=0.22$) did not have a significant effect on plant total biomass.

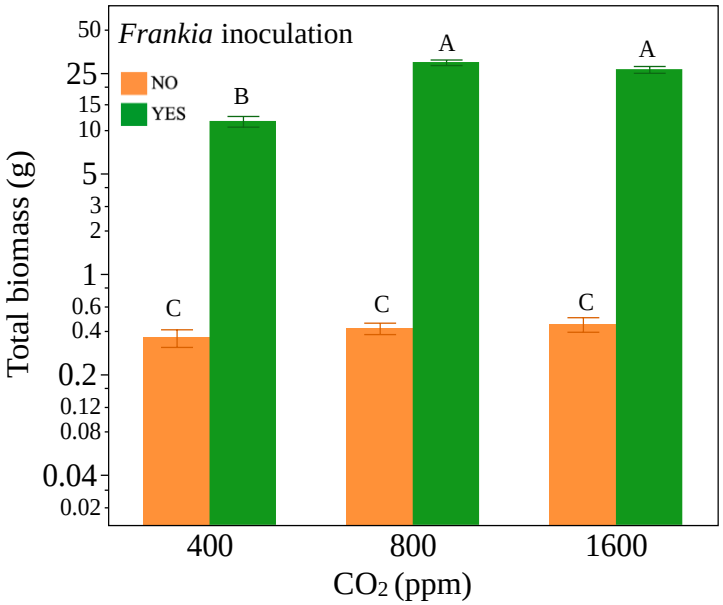

Figure 1: The effect of CO_2 level on *Alnus incana* ssp. *rugosa* total biomass with and without nitrogen-fixing nodules, averaged by non-herbivore and herbivore treatments. Bars with the same letters are not significantly different according to a Tukey's HSD Test. Bars are means with standard errors.

2.2. Leaf C:N ratio

CO_2 level and *Frankia* inoculation had an interactive effect on leaf C:N ratio ($F = 3.41$, $P = 0.04$, Figure 2). At 400 ppm, the C:N ratio in non-inoculated plans was twice as high as inoculated plants. Increasing CO_2 levels significantly increased plant leaf C:N ratio, but more in *Frankia*-inoculated plants, such that at 800 and 1600 ppm CO_2 there was no difference between plants with and without nitrogen-fixing nodules.

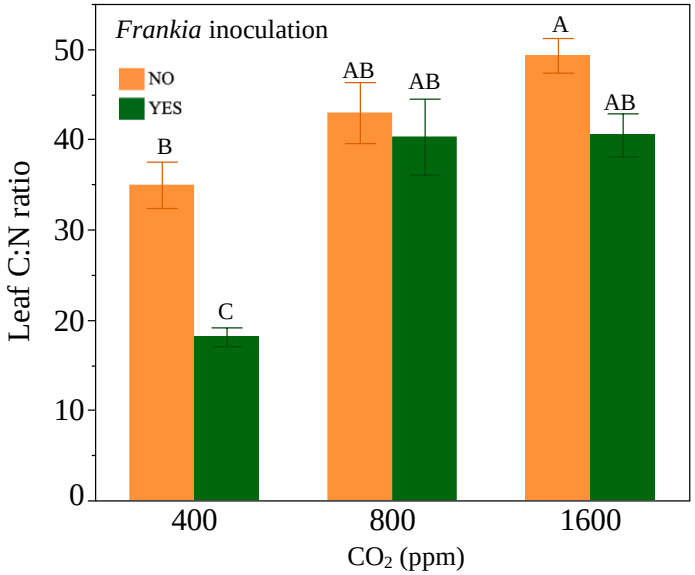

Figure 2: The effect of *Frankia* inoculation and CO_2 level on leaf C:N ratio. Green colour bars represented *Frankia*-inoculated plants, and orange color bars represented that plants were not. Bars with the same letters are not significantly different according to a Tukey's HSD Test. Bars are means with standard error.

2.3. Nitrogen fixation

After plants were fed on and one week after artificially damaged, nitrogen fixation per plant was higher in plants grown at a higher CO_2 level, regardless of whether or not they were part of the herbivory treatment ($F = 14.73$, $P < 0.0001$), increasing from 14.73 ± 1.01 μmol C_2H_4 h^{-1} $plant^{-1}$ at 400 ppm CO_2, to 20.59 ± 1.91 and 27.18 ± 1.41 μmol C_2H_4 h^{-1} $plant^{-1}$ at 800 and 1600 ppm CO_2, respectively (Figure S2). At the time of harvest there was no effect of CO_2 level ($F = 0.84$, $P = 0.43$) or herbivory ($F = 0.00$, $P = 0.99$,) on rates of fixation per plant, but the rate had dropped to 1.8 ± 0.1 μmol C_2H_4 h^{-1} $plant^{-1}$ averaged over all treatments. When N fixation rate at the time of harvest was normalized to plant mass (not shown) ($F = 9.06$, $P = 0.0003$) or leaf mass ($F = 7.86$, $P = 0.0009$), the rate decreased with increasing CO_2 level (Figure 3), with no effect of herbivore damage ($F = 0.28$, $P = 0.59$) or interaction between herbivore damage and CO_2 level ($F = 0.81$, $P = 0.44$). This was reflected in patterns of plant mass allocation to nodules which decreased by 32%, from 2.20 ± 0.08 % of total mass at 400 ppm CO_2 to 1.60 ± 0.13 and 1.50 ± 0.07 % at 800 and 1600 ppm CO_2 respectively ($F = 23.11$, $P < 0.0001$), with no effect of herbivory ($F = 1.97$, $P = 0.16$), or interaction between CO_2 level and herbivory ($F = 1.5457$, $P = 0.2110$). Stable isotope analysis indicated that all inoculated plants got most of their nitrogen from symbiotic N fixation, with a mean value of 98.56% ± 0.38%. Consequently, CO_2 levels ($F = 0.27$, $P = 0.76$) did not affect the proportion of nitrogen in the inoculated plants derived from fixation.

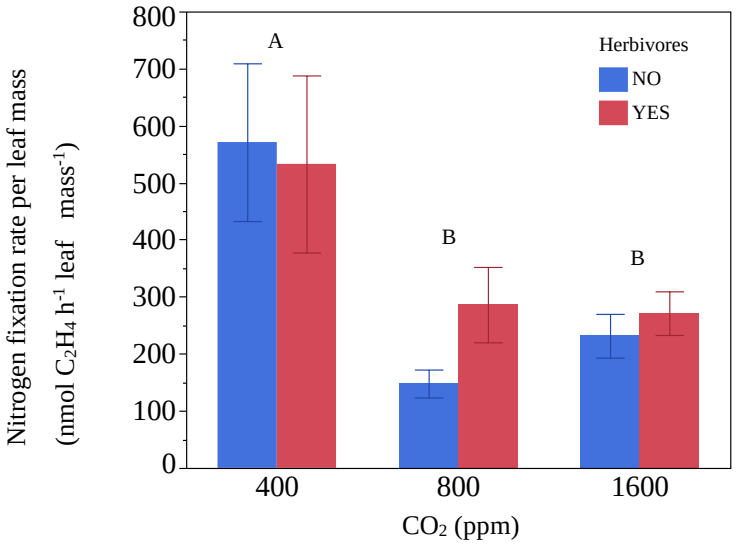

Figure 3: The effect of CO_2 level and herbivores on nitrogen fixation rate per leaf mass at harvest. Blue bars indicated plants without herbivore damage, and red bars indicated with herbivores damage. Different letters indicate significant differences between CO_2 levels. There was no difference between herbivore and non-herbivore treatments. Bars are means with standard error.

2.4. Leaf damage and antiherbivore compounds

After five days of feeding, more leaves on non-inoculated plants (18.24% ± 3.22%) were damaged on than nodulated plants (12.97% ± 1.30%, F = 3.49, P = 0.06) regardless of the CO_2 level. Increased CO_2 level had no effect on the leaf damage (F = 1.48, P = 0.23).

Elevated CO_2 increase leaf total phenolic concentration by the end of the growth period (F = 27.06, P < 0.0001, for the CO_2 effect, Figure 4), increasing by 35% at 1600 ppm CO_2 compared with 400 ppm and 800 ppm CO_2 treatments. Phenolic concentration also varied between herbivory treatments and plants with and without nitrogen-fixing nodules (F= 10.00, P = 0.003, for the interaction effect), and there was no three-way interaction between the treatments (F = 1.98, P = 0.15). We therefore examined the effect of *Frankia* inoculation and herbivore exposure at each CO_2 level separately. At 800 ppm CO_2, plants that were both able to fix nitrogen and exposed to herbivores had 18% higher phenolic concentration than nitrogen-fixing plants not exposed to herbivores or non-inoculated plants exposed to herbivores. At the other two CO_2 levels, there was no effect of *Frankia* inoculation or herbivory exposure on leaf phenolic levels.

Specific polyphenol oxidase (PPO) activity was 84.2 ± 21.7 units min^{-1} mg^{-1} protein averaged across all treatments, and specific peroxidase (POD) activity was 11.0 ± 3.0 units min^{-1} mg^{-1} protein, and the treatments had no effect on the level of these enzyme activity. There was no relationship between antiherbivore enzyme activities and the degree of leave damage (F = 1.54, P = 0.22). We also found no effect of the treatments on PPO acitivity when expressed on a leaf mass basis.

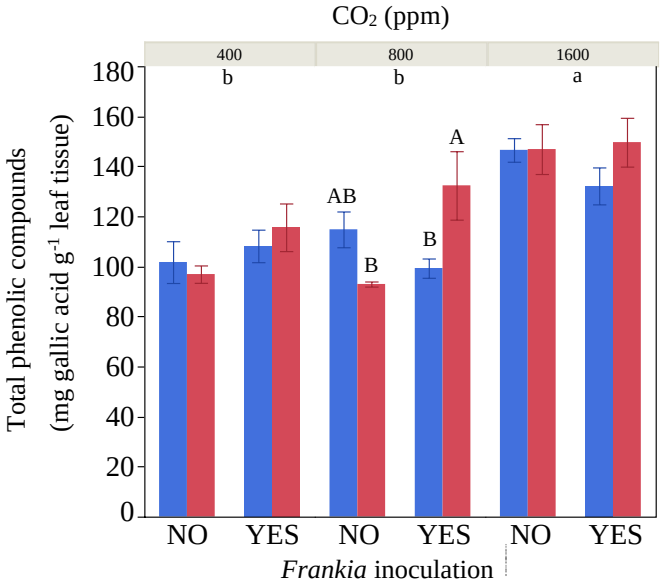

Figure 4: The effect of CO_2 level, *Frankia* inoculation and herbivore exposure on leaf total phenolic compounds. Blue bars represent plants without herbivores, and red bars represent plants exposed to herbivores. Different small letters indicate significant differences among the three levels of CO_2, averaged by *Frankia* and herbivore treatments. The different capital letters indicate significant differences between *Frankia* and herbivore exposure treatments within a CO_2 level. Bars are means with standard error.

2.5. Choice experiment

The leaf consumption area was log transformed due to the large differences in variation between treatments. Averaged across all CO_2 levels, herbivores preferred to feed on nitrogen-fixing plant leaves instead of leaves from non-nodulated plants ($F = 74.14$, $P < 0.0001$, for the inoculation effect). However, there was an interactive effect between CO_2 level and *Frankia* inoculation on leaf consumption ($F=4.80$, $P=0.01$, Figure 5). Herbivores showed greater preference for leaves from inoculated plants that were raise at either 400 or 1600 ppm CO_2, but there was no significant difference in the consumption of leaves between inoculated or non-inoculated plants grown at 800 ppm CO_2. The greatest leaf consumption occurred on leaves from nitrogen-fixing plants raised at 1600 ppm CO_2. There was no significant difference in the amount of leaf tissue consumed between non-inoculated plants raised at different levels. Pooling *Frankia* inoculated and non-inoculated plants together, herbivores consume almost twice the mass leaf tissues grown at 1600 ppm CO_2 compared with those grown at 800 ppm and 400 ppm ($F = 3.25$, $P = 0.06$, for a one-way ANOVA).

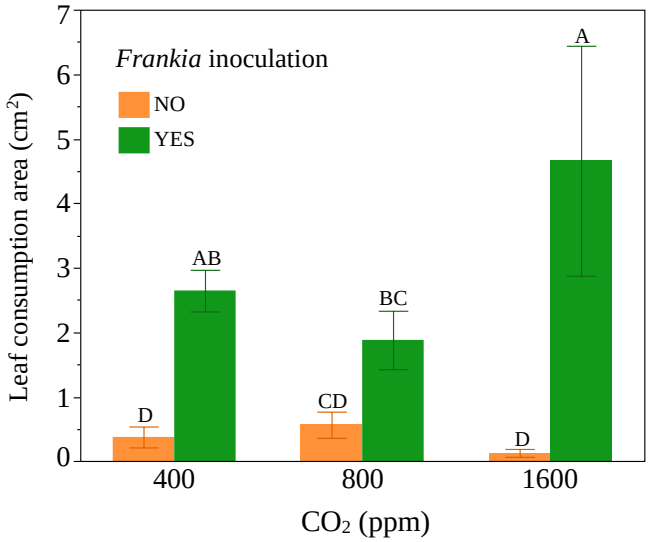

Figure 5: Insects consumption area on non-inoculated and inoculated plant leaves for one day at three levels of CO_2 (400 ppm, 800 ppm, 1600 ppm) at week 13. Bars with the same letters are not significantly different according to a Tukey's HSD test. Bars are mean value with standard error (SE). P = 0.01, F = 4.80.

3. Discussion

Many plants benefit from small increases in atmospheric CO_2 by increasing their photosynthetic rate, which boosts growth and yield [25,26]. Without nodules, our plants performed extremely poorly in soil from the field where alders are usually found. Consequently, only nodulated plants showed a growth increase with an increase in atmospheric CO_2. The lack of an increase in growth from 800 to 1600 ppm CO_2 and predicted peak in growth at around 1100 ppm CO_2 is consistent with other findings [16,27]. A review paper concluded that elevated CO_2 stimulated plant growth was dependent on soil nutrients availability [28]. The authors reported that under high soil N elevated CO_2 increased aboveground plant growth by 20.1%, while the response to elevated CO_2 was lower (+8.4%) under low soil N availability. Our study suggests that plants growing under present CO_2 conditions are not adapted to the CO_2 level under which symbiotic nitrogen fixation evolved, probably due to the essential nutrients deficeincy. It has also been suggested that elevated CO_2 accelerates leaf senescence due to an increased C:N ratio [29] or reactive oxygen species production and lower antioxidant enzymatic activity [30].

In our study, both future and ancient CO_2 levels increased leaf tissue C:N ratio, regardless of the ability of the plants to fix nitrogen. So, the growth conditions in this experiment do not support our first prediction that nitrogen-fixing plants can balance their C:N ratio when CO_2 levels increase to the levels we used. Previous studies have shown that non-nitrogen fixing plants tissue C:N ratio increased under elevated CO_2 (720 ppm) when plants were grown in low (2.2 g N m^{-2} yr^{-1}) or intermediate levels (6.7 g N m^{-2} yr^{-1}) of N availability [31]. The inability of nitrogen-fixing plants to maintain their C:N ratio that we found can be attributed to a reduced biomass allocation to nodules and, consequently, a decrease in the amount of nitrogen fixed per leaf mass under elevated CO_2. There are contradictory reports of the effects of elevated CO_2 on nitrogen fixation and plant N content [32]. Some studies show small increases in CO_2 (550 ppm) stimulates symbiotic N fixation [33], with up to a doubling of the rate of nitrogen fixation in black locust (*Robinia pseudoacacia*) at 700 ppm CO_2 [7]. In this latter study, plants were fertilized weekly with Hoagland-based nutrient solution for over one year, so other essential nutrients were not a limiting factor for nitrogen fixation. However, in natural ecosystems, nitrogen fixation may decrease in the long term with

increasing CO_2 due to the limitation of essential elements, especially P, Mo and Fe [34,35]. Hungate et al. [36] found that N fixation at elevated CO_2 in *Galactia elliottii* Nutt. in scrub-oak vegetation in central coastal Florida increased during the first year but then declined by the third year and subsequent years. This was due to Molybdenum becoming limited under elevated CO_2, which is a required cofactor for nitrogenase. Edwards et al. [37] also found that elevated CO_2 (700 ppm) had no effect on nitrogen fixation in white clover (*Trifolium repens*) with a low phosphate application (0.04 mM), but increased with a high phosphate application (1.0 mM). A reviewe paper summarized that biological nitrogen fixation did not increase under elevated CO_2 when no mineral nutrients were supplied, while nirogen fixation was significantly increased (+51%) by elevated CO_2 when additional non-N mineral nutrients were supplied [28]. Our study found that whole-plant nitrogen fixation rate increased under elevated CO_2 at week 14-15, but dropped by week 19 regardless of CO_2 levels. As whole plant rates of N fixation are a function of plant mass, the early differences in fixation may simply be due to differences in plant mass. Still, decreases over time suggest increasing nutrient limitation on nitrogen fixation since plants were stopped fertilization after grown 12 weeks. It is possible that the nutrient-poor soil we used prevented nodulated plants from being able to maintain their tissue C:N ratio. It is also likely that in the future, nitrogen-fixing plants will find themselves in conditions with less available nutrients as the increased carbon content of litter can result in a general immobilization of plant nutrients [38].

We did find evidence for our second prediction. Carbon-based antiherbivore compounds did increase with increasing CO_2. Also, N fixing plants did maintain their levels of N based antiherbivore compounds, even though the N fixing plants were not able to maintain their C:N ratio. We also found that having the ability to fix nitrogen increased total phenols when the plants were exposed to herbivores at 800 ppm CO_2. This supports other studies showing that nitrogen fixation increases inducible herbivore defence [23,39] and may provide some feeding deterrence at future CO_2 levels but not at ancient levels.

Our results also confirmed the third prediction that plants get less leaf damage when leaf tissue accumulated more total phenolic compounds at elevated CO_2, but we did not find that the nitrogen-based antiherbivore compound production was related to the degree of plant damage from herbivores. Total phenolic compounds play a major role in host resistance to herbivores [40–42] because they can bind to insects' digestive enzyme and interfere with animal digestion [43]. The defence mechanism can be present constitutively or induced after damage by the herbivores [39]. Several studies have shown that plants exhibit many inducible defence mechanisms [23,39]. They can both deter feeding and retard insect development [44]. Previous studies have also shown that total phenolic compounds of maize (*Zea mays*) increased under elevated CO_2 [45]. Previous studies have also shown that plants can accumulate more N-based secondary compounds (e.g., polyphenol oxidase, peroxidase) to defend against herbivore damage [39,46]. Polyphenol oxidase and peroxidase are the major anti-nutritive enzymes that catalyze the oxidation of phenolics to quinones, which is a defence mechanism in plants against herbivore damage since quinones bind to leaf protein and inhibit protein digestion in herbivores [39,46]. Our results support the previous study that *Alnus* exhibited the inducible defence mechanism in response to herbivore feeding by maintaining polyphenol oxidase and peroxidase activity under elevated CO_2.

The choice experiment showed that herbivores generally prefer to eat the nodulated plants rather than non-nodulated, confirming our 4th prediction. Herbivores also increased consumption at higher CO_2, confirming our 5th prediction that herbivores consume more when food quality goes down. However, there was no difference in herbivore preference between nodulated and non-nodulated plant leaves when grown at 800 ppm CO_2. This may be related to the fact that at this CO_2 concentration, nitrogen-fixing plants had a higher concentration of phenolic compounds when they had previously been fed on, suggesting they can have a greater induced antiherbivore response than non-nodulated plants. This result also supports a previous study suggesting that plant leaf defences, not leaf nutritional content, are the dominant driver of herbivore preference [47](Taylor and Ostrowsky 2019).

4. Materials and Methods

4.1. Plant growth condition and treatments

Seedlings of speckled alder (*Alnus incana* ssp. *rugosa*) were raised in growth chambers with 16-h day time/8-h night time (22 °C /18 °C, day/night). The plants were grown in a mix of field soil from a *Pinus banksiana* forest stand that contains *Alnus viridis* ssp. *crispa*, mixed with an equal volume of Turface. A previous study has shown that the soil from the site has an inorganic N level of 10.2 ± 0.6 mg/kg and an extractable phosphate level of 0.98 ± 0.33 mg/kg [48]. Plants were fertilized with 1/16 N-Rorison nutrient solution containing 0.125 mM $Ca(NO_3)_2$, 1 mM $CaCl_2$, 1 mM K_2HPO_4, 1 mM $MgSO_4$, 0.0534 mM Fe-EDTA, 0.009 mM $MnSO_4$, 0.0045 mM H_3BO_3, 0.001 mM Na_2MoO_4, 0.0015 mM $ZnSO_4$, and 0.0015 mM $CuSO_4$ [49] once a week for total of 12 weeks and stopped fertilization before the choice experiment described below. Half of the plants were inoculated with the nitrogen-fixing symbiont *Frankia*, which came from crushed root nodules collected from wild plants. The non-inoculated plants received the same dose of inoculum, which was first autoclaved. Plants were divided among six growth chambers, with two chambers each receiving one of three atmospheric CO_2 levels: 400, 800, and 1600 ppm, representing ambient, future and Cretaceous era atmospheric levels, respectively, following Murray et al. [50]. There were 30 plants per each combination of inoculation treatment and CO_2 level. In each chamber, carbon dioxide levels were continuously monitored and controlled with a CO_2 sensor connected to a microcontroller, which controlled a CO_2 injection system [51].

4.2. Choice experiment

At week 13 after the start of the CO_2 treatments, a choice experiment was set up to determine whether insects prefer non-nodulated or nodulated plants grown at each CO_2 level. We randomly paired one leaf from an inoculated plant with one leaf from a non-inoculated plant grown at the same CO2 level. Each pair of leaves were placed into a petri dish with moistened filter paper and along with one white-marked tussock caterpillar (*Orgyia leucostigma*) larva. The larvae were raised from eggs obtained from the Great Lakes Forestry Centre (Natural Resources Canada) on their artificial diet at 22 °C on a 12h light: 12 h dark cycle for one month in room level (*ca.* 400 ppm) CO_2. Insects were starved for 48h before placing them into the petri dishes. Petri dishes were placed under the same conditions where larvae were raised. Nine inoculated and nine non-inoculated leaves were collected from each chamber, for a total of 18 replicates at each level of CO_2. Leaf areas were measured before and after 24 hours of insect feeding to determine insect preference.

4.3. Herbivores treatment

After the choice experiment, half of the plants in each combination of inoculation treatment and CO_2 level (15 plants) were randomly assigned to an herbivore exposure treatment of *O. leucostigma*, raised and starved as above. The rest were non-herbivore controls. Since it was not possible to control insect movement, all plants exposed to herbivores were moved to one chamber at each CO_2 level for the feeding period and then moved back after feeding. Each plant was exposed to an average of 5 insects (for a total of 145 larvae in each chamber) for five days without controlling the insect's movement. After the insects were removed, the proportion of leaves showing signs of insect feeding was assessed. Then, half of each plant's leaves were artificially removed to standardize plant damage. The plants were grown for another six weeks after artificial leaf removal and then harvested.

4.4. nitrogen fixation

Nitrogen fixation rate was measured after leaves were damaged (at week 14-15) and at the time of the harvest (week 20) using acetylene reduction assays [49]. For the pre-harvest assays, the acetylene reduction rate was expressed per plant. At the time of harvest, the acetylene reduction rate was also calculate per total mass and leaf dry mass, to give an index of the amount of N fixed per mass of photosynthetic tissue. After harvest, dry plant leaf tissues were ground in a ball mill. Leaf C, N content, and their isotopic ratios were measured by the Stable Isotope Facility at the University of California,

Davis. The percentage of nitrogen fixation derived from air (%Ndfa) was calculated using a modification of the equation following Boddey et al. [52]:

$$\%\text{Ndfa} = \frac{(\delta^{15}N_{reference} - \delta^{15}N_{fixing\ plant})}{(\delta^{15}N_{reference} - \text{B})} \times 100$$

Where $\delta^{15}N_{reference}$ is the level of $\delta^{15}N$ in the uninoculated plants , $\delta^{15}N_{fixing\ plant}$ is the level of $\delta^{15}N$ in the inoculated plants (*Alnus incana* ssp. *rugosa*) and B is the $\delta^{15}N$ of nitrogen fixing plants that are fully dependent upon symbiotic N_2 fixation and sampled at the same growth stage.

4.5. C-based antiherbivore compounds

The C-based antiherbivore compounds were analyzed at the time of harvest. Total phenolic compounds were determined using the Folin-Ciocalteu method [53]. In brief, 0.01 g dry leaf powder was incubated in the dark for 24 hours in 10 mL of 40% ethanol. After centrifuging at 5000 RPM for 10 minutes, 1 mL of supernatant was mixed with 0.5 mL 50% Folin-Ciocalteu reagent, incubated for 3 minutes and then incubated in the dark for 30 min with 1 mL of 5% Na_2CO_3. The absorbance was then measured at 750 nm and compared to a standard curve prepared with known concentrations of gallic acid.

4.6. N-based antiherbivore compounds

The N-based antiherbivore compounds were analyzed after leaf damage. The polyphenol oxidase and guaiacol peroxidase assays were only performed on *Frankia* inoculated plants since the non-inoculated plants did not produce enough tissue to conduct all assays. Polyphenol oxidase and guaiacol peroxidase activity were measured relative the protein content of leaves. Leaf protein content was determined with a Bio-Rad protein assay using the Bradford [54] method. In brief, 0.6 grams of frozen leaf tissue was ground with liquid N_2 and then homogenized in 10 mL extraction buffer consisting of 50 mM K-phosphate (pH 7.1), 1% PVP (polyvinyl-pyrrolidone), one mM EDTA (Ethylenediaminetetraacetic acid), and five mM ascorbic acid. The homogenate was centrifuged at 23,000 g for 20 min at 4 °C. The supernatant was used for both the protein and antiherbivore compound assays. The protein content was measured after adding the Bradford reagent, using BSA as a standard. Polyphenol oxidase and guaiacol peroxidase were measured following Tscharntke et al. [23]. For the polyphenol oxidase assay, 0.15 mL of supernatant was mixed with 1.1 mL of 50 mM potassium phosphate (pH 7.1) and 0.3 mL of 100 mM catechol. After two minutes, the absorbance at 420 was measured. One unit of polyphenol oxidase was defined as the amount of enzyme that caused an increase in the absorbance of 0.01 per minute, and specific activity was expressed as units min^{-1} mg $protein^{-1}$ [55]. The guaiacol peroxidase assay was performed by mixing 0.15 mL of supernatant with 1.2 mL of 50 mM K-phosphate (pH 7.1), 0.15 mL of 20 mM guaiacol, and 0.06 mL of 12.3 mM H_2O_2. The enzyme activity was calculated from the increase in the absorbance at 470 nm after 2 minutes [23]. One unit of activity was defined as the amount of enzyme that increases the optical density 470 by 0.01 per min. The specific enzyme activity was expressed as a change in optical density (ΔOD) per min per mg protein (units min^{-1} mg^{-1} protein).

4.7. Statistical analysis

Data were analyzed using JMP Pro 14. The data were analyzed using three-factor (CO_2 level, Frankia inoculation, and herbivory) ANOVA models followed by Tukey's HSD tests, with growth chamber nested within CO_2 treatments. The non-inoculation treatment was dropped from the model for the nitrogen fixation response variables, as non-inoculated plants did not fix nitrogen. We also explored the relationships between variables using linear and non-linear least squares models. Since the effect of the CO_2 level on a number of plant responses was not linear, quadratic models were also used to fit the data. Residual plots were used to determine if there are large differences in variation between treatments. If so, the data were log-transformed.

5. Conclusions

Our study found that nitrogen fixation cannot meet the higher N demand of plants when growth under 800 and 1600 ppm CO_2, the former being a CO_2 concentration that may occur in the future and the latter a level that occurred in the distant past. The increased leaf C:N ratio under elevated CO_2 is associated with higher total leaves phenol content. Like non-nitrogen fixing plants, nitrogen-fixing plants at present have likely evolved not to be able to acclimate ancient CO_2 levels. Nutrient limitation likely prevents the nitrogen fixation rate increasing under elevated CO_2. Future studies are needed to test the effect of ancient CO_2 on nitrogen fixation when other nutrients are not limited. Finally, field studies are required to examine the complex relationship between elevated CO_2, nitrogen fixation and herbivory.

Supplementary Materials: The following are available online at

Figure S1: The effect of elevated CO_2 on total biomass of nodulated/non-nodulated *Alnus incana* ssp. *rugosa*. ($P=0.027$, $F=14.81$ from quadratic fit). Non-linear quadratic fit line: $Y=15.99+0.017*X-4.16*10^{-5}*(X-933.33)^2$

Figure S2: Effect of elevated CO_2 and herbivores on nitrogen fixation rate per plant after herbivore and artificial damage (at week 14 and 15). Blue bars indicated nitrogen fixation rate without herbivore damage. Red bars indicated nitrogen fixation rate with herbivores damage. Bars are mean value with standard error (SE). Letters were from Tukey HSD Test. Different letters indicated a significant difference. There was no significant difference between herbivore and non-herbivore treatments.

Figure S3: By the end of week 10, nodulated plants' leaves started to show dark spots, which was similar to K deficiency symptoms in my previous study (a, c). At week 10, under elevated CO_2 (800 ppm and 1600 ppm), nodulated plants' leaves margin started to show reddish colour, which leads to leaf senescence faster (b). After plants grown for 19 weeks, elevated CO_2 accelerates both inoculated and non-inoculated plant leaf senescence (d). Plant leaf senescence was more severe at 1600 ppm compared with 800 ppm treatments (d).

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